REMARKS

Applicants respectfully request entry of the above amendments and reconsideration of the claims given the following arguments pursuant to 37 C.F.R. § 1.111.

1. Status of the Claims and Support for the Claim Amendments

The status of the claims upon entry of the present amendments stands as follows:

Pending Claims: 1-22

Rejected Claims: 1-22

Amended Claim: 1 and 19

Applicants amend claims 1 and 19 to more precisely recite the claimed subject matter. Support for the amendments can be found at least at (1) lines 4-14, page 2, and (2) line 33, page 2 to line 2, page 3 of the Specification ("... rice protein is generally denatured at temperatures above 75°C..."). Applicants do not believe that the amendments add prohibited subject matter that is unsupported in the Specification as filed.

The claims have been amended without prejudice to, or disclaimer of, the canceled subject matter. Applicants reserve the right to file a continuation or divisional application on any subject matter canceled by way of amendments.

2. Certified Priority Document(s)

Applicants respectfully request acknowledgement of the certified priority documents submitted July 21, 2006, with the Office's next communication.

3. Acceptance of Drawings

Applicants respectfully request the status regarding the acceptance of the drawings filed July 21, 2006, with the Office's next communication.

4. Rejection Under 35 U.S.C. § 102(b)

Grounds For Rejection

The Office rejects claims 1-5 and 10-11 under 35 U.S.C. § 102(b) as allegedly anticipated by **Mitchell** et al., U.S. Patent No. 4,892,242 ("Mitchell"). Office Action, page 2. Mitchell

allegedly discloses enzymatically hydrolyzing a rice substrate with a glucoamylase and an alpha-amylase at a temperature of 30°C to 100°C and at a pH of 3.5 to 7.5. *Id.*

Arguments

Applicants traverse the rejection to the extent it may be applied to the amended and unamended claims. To anticipate a claim, each claim element must be explicitly or inherently disclosed. *See In re Rijckaert*, 9 F.3d 1531, 1534, 28 U.S.P.Q.2d 1955, 1957 (Fed. Cir. 1993). Additionally, the prior art anticipates only when "the prior art ***necessarily*** functions in accordance with, or includes, the claim limitations." *Perricone v. Medicis Pharm. Corp.*, 432 F.3d 1368, 1375, 77 U.S.P.Q.2d 1321, 1326 (Fed. Cir. 2005) (emphasis added) (citing *MEHL/Biophile Int'l Corp. v. Milgraum*, 192 F.3d 1362, 1365, 52 U.S.P.Q.2d 1303, 1305 (Fed. Cir. 1999)).

The Office's rejection is unsupported. Claim 1 as amended recites, *inter alia*, (1) enzymatically hydrolyzing a rice substrate with a granular starch hydrolyzing (GSH) enzyme and a second starch hydrolyzing enzyme (*e.g.*, alpha amylase) at the claimed temperature and pH; and (2) obtaining a rice concentrate wherein the rice protein is ***not*** denatured. Mitchell does not disclose at least these two elements.

Applicants submit that "glucoamylase" and "granular starch hydrolyzing enzyme" (i.e., "raw starch hydrolyzing enzyme" and "raw starch digesting enzyme") are ***not*** interchangeable concepts. The claimed granular starch hydrolyzing enzyme "refers to an enzyme having the ability to hydrolyze starch in granular form." *See* Specification, lines 28-30, at page 9. However, ***not*** every glucoamylase is capable of hydrolyzing a granular starch substrate. *See, e.g.*, U.S. Patent No. 7,262,041, paragraph bridging columns 1-2.[1] Conversely, a GSH enzyme can be an enzyme other than a glucoamylase. A GSH enzyme can be, for example, an alpha

[1] "***Certain*** thermophilic and mesophilic fungi, and particularly strains of *Humicola grisea* and *Aspergillus awamori*, produce an enzyme having ***both glucoamylase activity and the ability to hydrolyze raw starch***. These glucoamylases are referred to as granular starch hydrolyzing enzymes (GSHE) and are also known in the art as raw starch hydrolyzing (RSH) enzymes. Additionally, while these enzymes will hydrolyze thinned starch hydrolyzate to glucose in a manner similar to other known glucoamylases, they frequently have a pH optimum in the range of 5.0 to 7.0 as compared to a pH optimum of less than 5.0 for widely used glucoamylase preparations (See, Tosi et al., (1993) *Can. J. Microbiol.*, 39: 846-851)." (Emphasis added).

amylase of fungal origin. *See, e.g.*, Matsubara et al., 37 J. BIOCHEM. MOL. BIOL. 429 (2004) (enclosed as **EXHIBIT I**).

Mitchell at best may disclose (1) hydrolyzing a rice slurry with a bacterial alpha-amylase at 80°C for 30 minutes and then at 100°C for 15 minutes; (2) cooling the slurry to 60°C; and (3) treating the slurry at 60°C with a beta-amylase and a glucoamylase for two to three hours. *See* Examples 1-2, columns 8-9. There is no evidence on the record, or adduced by the Office, that the glucoamylase used in Mitchell necessarily would have the granular starch hydrolyzing activity. Thus, Mitchell fails to disclose, explicitly or inherently, at least the claimed GSH enzyme.

Claim 1, by reciting the properties of the resulting rice protein concentrate, is further distinguishable over Mitchell's process. Mitchell's process involves liquefying a rice slurry at 80°C for 30 minutes and then at 100°C for 15 minutes. Under Mitchell's conditions, the rice protein necessarily is denatured. *See* Specification, lines 4-14, at page 2 ("... rice protein is generally denatured at temperatures ***above 75°C***...") (emphasis added).

As Mitchell fails to disclose each and every claim element, claim 1 is novel. Dependent claims 2-5 are likewise novel for at least the same reasons. Accordingly, Applicants respectfully request withdrawal of the rejection and allowance of the claims.

5. Rejections Under 35 U.S.C. 103(a)

5.1. Claim 6

Grounds For Rejection

The Office rejects claim 6 under 35 U.S.C. § 103(a) as allegedly obvious over **Mitchell** in view of **Radford** et al., U.S. Patent No. 5,834,191 ("Radford"). Office Action, page 4. The alleged teachings and deficiencies of Mitchell are discussed above. The Office admits that Mitchell does not teach "the enzyme having GSH activity as being obtained from the heterologous expression of a GSH enzyme in a *Trichoderma* strain or an *Aspergillus* stain." *Id.* Radford allegedly teaches heterologous expression of hydrolytic enzymes, such as glucoamylase, in *Aspergillus* strains. *Id.* It allegedly would have been obvious to one having ordinary skill in the art to incorporate the enzyme produced in Radford into the process of Mitchell. *Id.*

Arguments

Applicants traverse. "[O]bviousness requires a suggestion of ***all*** limitations in a claim." *CFMT, Inc. v. Yieldup Int'l Corp.*, 349 F.3d 1333, 1342, 68 U.S.P.Q.2d 1940, 1947 (Fed. Cir. 2003) (emphasis added). Additionally, once the scope and content of the prior art are determined, the relevant inquiry is whether the prior art suggests the invention, and whether one of ordinary skill in the art would have had a reasonable expectation that the claimed invention would be successful. *In re Vaeck*, 947 F.2d 488, 493, 20 U.S.P.Q.2d 1438, 1442 (Fed. Cir. 1991).

The Office's rejection is unsupported for at least the following reasons. Claim 6, by depending directly upon claim 1 and incorporating all elements from claim 1, recites *inter alia* (1) enzymatically hydrolyzing a rice substrate with a granular starch hydrolyzing (GSH) enzyme and a second starch hydrolyzing enzyme (*e.g.*, alpha amylase) at the claimed temperature and pH; and (2) obtaining a rice concentrate wherein the rice protein is ***not*** denatured.

Mitchell at best may teach (1) hydrolyzing a rice slurry with a bacterial alpha-amylase at 80°C for 30 minutes and then at 100°C for 15 minutes; (2) cooling the slurry to 60°C; and (3) treating the slurry at 60°C with a beta-amylase and a glucoamylase for two to three hours. *See* Examples 1-2, columns 8-9. As discussed in Section 4 *supra*, "glucoamylase" and "granular starch hydrolyzing enzyme" (i.e., "raw starch hydrolyzing enzyme" and "raw starch digesting enzyme") are ***not*** interchangeable concepts, and Mitchell's process results in denaturation of the rice protein. Thus, Mitchell fails to teach at least (1) hydrolyzing a rice substrate with a GSH enzyme and (2) obtaining a rice protein concentrate having the claimed properties. Radford is relied upon for its purported teachings of expressing a modified / heterologous glucoamylase gene in filamentous fungi. *See, e.g.*, Radford, Abstract ("The invention relates to a method and recombinant means for engineering the production of heterologous peptides in filamentous fungus."). Radford does not teach (1) hydrolyzing a rice substrate with a GSH enzyme, and (2) avoiding the denaturation of the rice protein. Thus, Radford fails to cure at least Mitchell's defects. Mitchell and Radford, alone or when viewed in combination, fail to teach or suggest all claim elements. Without all claim elements taught, there can be no expectation of success to practice the claimed methods.

Given at least the above arguments, claim 6 is nonobvious over the cited references. Accordingly, Applicants respectfully request withdrawal of the rejection and allowance of claim 6.

5.2. Claims 7-9

The Office rejects claims 7-9 under 35 U.S.C. § 103(a) as allegedly obvious over **Mitchell** in view of **Puski** et al., U.S. Patent No. 4,830,861 ("Puski"). Office Action, page 4. The alleged teachings and deficiencies of Mitchell are discussed above. The Office admits that Mitchell does not teach purifying the rice protein concentrate. *Id.* Puski allegedly teaches (1) separating rice syrup from high protein rice flour, which allegedly qualifies as a purification step; (2) drying the rice protein concentrate; and (3) obtaining a rice protein concentrate having a protein content of 44%. *Id.*, at 5. It allegedly would have been obvious to one having ordinary skill in the art to incorporate the purification step of Puski into the process of Mitchell. *Id.*

Each of claims 7-9 depends directly upon claim 1, and thus incorporates all elements from claim 1. As discussed in Sections 4 and 5.1 *supra*, Mitchell fails to teach at least (1) hydrolyzing a rice substrate with a GSH enzyme, and (2) obtaining a rice protein concentrate having the claimed properties. Puski is relied upon for its purported teachings of purifying a rice protein concentrate. Puski at best may teach hydrolyzing a rice flour slurry with a heat-stable alpha amylase (Termamyl or Takalite) at 75°C to 100°C (preferably at 90°C). *See* Puski, column 6, lines 57-65. The heat-stable alpha amylase does not have GSH activity—Puski does not teach hydrolyzing a rice substrate with a GSH enzyme. Additionally, Puski's process (e.g., 75°C to 100°C treatment) results in the denaturation of the rice protein. Thus, Puski fails to cure at least Mitchell's defects. Mitchell and Puski, alone or when viewed in combination, fail to teach or suggest all claim elements. Without all claim elements taught, there can be no expectation of success to practice the claimed methods.

Given at least the above arguments, claims 7-9 are nonobvious over the cited references. Accordingly, Applicants respectfully request withdrawal of the rejection and allowance of the claims.

5.3. Claims 12-16 and 18

The Office rejects claims 12-16 and 18 under 35 U.S.C. § 103(a) as allegedly obvious over **Mitchell** in view of **Euber** et al., U.S. 4,990,344 ("Euber"). Office Action, page 5. The alleged teachings and deficiencies of Mitchell are discussed above. The Office admits that Mitchell does not teach repeating the steps of enzymatically hydrolyzing the rice protein concentrate and separating the fractions. *Id.*, at 6. Euber allegedly teaches "a similar rice protein purification process ... wherein 'protein levels approaching 90% to 100% can be achieved with increased water to rice ration and wash steps.'" *Id.* It allegedly would have been obvious to one having ordinary skill in the art to repeat the steps of enzymatically hydrolyzing the rice protein concentrate and separating the fractions to obtain a high-purity protein product as taught in Euber. *Id.*

Each of claims 12-16 and 18 depends directly or indirectly upon claim 1, and thus incorporates all elements from claim 1. As discussed in Sections 4 and 5.1 *supra*, Mitchell fails to teach at least (1) hydrolyzing a rice substrate with a GSH enzyme, and (2) obtaining a rice protein concentrate having the claimed properties. Euber is relied upon for its purported teachings of purifying a rice protein concentrate to reach a protein level of 90% to 100%. Euber at best may teach digesting a rice raw material with a thermostable alpha amylase (Termamyl or Takalite) at 90°C to 95°C for 40 minutes. *See* Euber, column 8, lines 7-24. The thermostable alpha amylase does not have GSH activity—Euber does not teach hydrolyzing a rice substrate with a GSH enzyme. Additionally, Euber's process (e.g., 90°C to 95°C for 40 minutes) results in the denaturation of the rice protein. Thus, Euber fails to cure at least Mitchell's defects. Mitchell and Euber, alone or when viewed in combination, fail to teach or suggest all claim elements. Without all claim elements taught, there can be no expectation of success to practice the claimed methods.

Given at least the above arguments, claims 12-16 and 18 are nonobvious over the cited references. Accordingly, Applicants respectfully request withdrawal of the rejection and allowance of the claims.

5.4. Claim 17

The Office rejects claim 17 under 35 U.S.C. § 103(a) as allegedly obvious over **Mitchell** in view of **Euber** as applied to claim 12, and further in view of **Mihara** et al., U.S. Patent No. 3,852,504 ("Mihara"). Office Action, page 7. The alleged teachings and deficiencies of Mitchell and Euber are discussed above. The Office admits that Mitchell and Euber do not teach an animal feed formulation comprising the rice protein concentrate. *Id.* Mihara allegedly teaches (1) a rice protein product "that is highly nutritious"; and (2) "a crude rice fiber product containing some protein that is 'sufficiently nutritious to be sued as an animal feed.'" *Id.* It allegedly would have been obvious to one having ordinary skill in the art to incorporate a high-purity rice protein product in animal feed. *Id.*

Claim 17 depends directly upon claim 12, and incorporates all elements from claim 12. Mitchell and Euber fails to render claim 12 obvious for at least the reasons discussed in Section 5.3 *supra*. Mihara is relied upon for its purported teachings of including a rice protein product in animal feed. Mihara does not teach (1) hydrolyzing a rice substrate with a GSH enzyme, and/or (2) obtaining a rice protein concentrate having the claimed properties. Thus, Mihara fails to cure at least these defects of Mitchell and Euber. Mitchell, Euber, and Mihara, alone or when viewed in combination, fail to teach or suggest all claim elements. Without all claim elements taught, there can be no expectation of success to practice the claimed methods.

Given at least the above arguments, claim 17 is nonobvious over the cited references. Accordingly, Applicants respectfully request withdrawal of the rejection and allowance of claim 17.

5.5. Claims 19-22

The Office rejects claims 19-22 under 35 U.S.C. § 103(a) as allegedly obvious over **Mitchell** in view of **Puski**, **Euber**, and **Mihara**. Office Action, page 8. The alleged teachings and deficiencies of Mitchell, Puski, Euber and Mihara are discussed above. The Office admits that Mitchell does not teach (1) hydrolyzing 60% of the starch in the rice substrate; and (2) adding the rice protein concentrate to an animal feed. *Id.* Puski allegedly teaches hydrolyzing starch to achieve acceptable high protein rice flour protein level. *Id.* Euber allegedly teaches a rice protein purification process yielding protein levels approaching 90% to 100%. *Id.* It

allegedly would have been obvious to one having ordinary skill in the art to (1) repeat the enzymatic hydrolysis to increase purity; and (2) incorporate a high-purity rice protein product in animal feed. *Id.*, 8-10.

Applicants traverse the rejection to the extent it may be applied to the amended and unamended claims. The Office's rejection is unsupported, because not all claim elements are taught for at least the following reasons. Claim 19 as amended recite *inter alia* (1) hydrolyzing a rice substrate at the claimed temperature with a ***combination*** of (i) a starch hydrolyzing enzyme (*e.g.*, an alpha amylase) and (ii) a granular starch hydrolyzing (GSH) enzyme; and (2) obtaining a rice concentrate having the claimed properties. Mitchell's deficiencies are discussed in Sections 4 and 5.1 *supra*. Mitchell at least fails to teach (1) using a combination of a starch hydrolyzing enzyme and a GSH enzyme to hydrolyze a rice substrate; and (2) obtaining a rice protein concentrate having the claimed properties. As discussed in Sections 5.2-5.4, Puski, Euber and Mihara fails to cure at least these defects of Mitchell. Thus, Mitchell, Puski, Euber and Mihara, alone or when viewed in combination, fail to teach or suggest all claim elements. Without all claim elements taught, there can be no expectation that the presently claimed methods would have worked predictably.

Given at least the above arguments, claim 19 is nonobvious over the cited references. Dependent claims 20-22 are likewise nonobvious for at least the same reasons. Accordingly, Applicants respectfully request withdrawal of the rejection and allowance of the claims.

CONCLUSION

The claims as amended are believed in condition for allowance, which is respectfully requested. If there are any other fees due in connection with the filing of this reply, please charge the fees to Deposit Account No. 50-0573 [048452-0070-00-US-475169]. If a fee is required for an extension of time under 37 C.F.R. § 1.136 not accounted for above, such an extension is requested and the fee should also be charged to this Deposit Account. If the Examiner has any questions about Applicants' response, please contact Steven G. Bacsi at (650) 846-5828 (**Attorney Docket No. GC830-US**). The undersigned representative signs in his capacity under 37 C.F.R. § 1.34.

Respectfully submitted,

Dated: October 4, 2011 By: _____

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EXHIBIT I

Matsubara et al., 37 J. BIOCHEM. MOL. BIOL. 429 (2004), *available at* http://www.jbmb.or.kr/fulltext/jbmb/view.php?vol=37&page=429

Journal of Biochemistry and Molecular Biology, Vol. 37, No. 4, July 2004, pp. 429-438

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Molecular Cloning and Determination of the Nucleotide Sequence of Raw Starch Digesting α-Amylase from *Aspergillus awamori* KT-11

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Complementary DNAs encoding α-amylases (Amyl I, Amyl III) and glucoamylase (GA I) were cloned from *Aspergillus awamori* KT-11 and their nucleotide sequences were determined. The sequence of Amyl III that was a raw starch digesting α-amylase was found to consist of a 1,902 bp open reading frame encoding 634 amino acids. The signal peptide of the enzyme was composed of 21 amino acids. On the other hand, the sequence of Amyl I, which cannot act on raw starch, consisted of a 1,500 bp ORF encoding 499 amino acids. The signal peptide of the enzyme was composed of 21 amino acids. The sequence of GA I consisted of a 1,920 bp ORF that encoded 639 amino acids. The signal peptide was composed of 24 amino acids. The amino acid sequence of Amyl III from the N-terminus to the amino acid number 499 showed 63.3% homology with Amyl I. However, the amino acid sequence from the amino acid number 501 to C-terminus, including the raw-starch-affinity site and the TS region rich in threonine and serine, showed 66.9% homology with GA I.

Keywords: *Aspergillus awamori* KT-11, α-Amylase, Glucoamylase, Raw starch digestion

Introduction

α-Amylases (α-1,4 glucan-4-glucanohydrolase, EC 3.2.1.1) are widely distributed in animals, plants and microorganisms and catalyse the hydrolysis of α-1,4-glucosidic linkages of starch, glycogen and related polysaccharides to produce the α-anomeric form of glucose and oligosaccharides. The raw-starch-binding and raw-starch-digesting abilities of glucoamylase G1 from *Aspergillus niger* have been intensively studied (Ueda, 1981; Takahashi *et al.*, 1985; Belshaw and Williamson, 1993; Stoffer *et al.*, 1993; Semimaru *et al.*, 1995). Although some α-amylases also have the ability to digest raw starch, few researches have been reported on the digestibility of raw starches by α-amylases (Taniguchi *et al.*, 1983; Hayashida *et al.*, 1988; Mizokami, 1988; Kin *et al.*, 1989; Monma *et al.*, 1989; Hayashida *et al.*, 1990; Punpeng *et al.*, 1992; Iefuji *et al.*, 1996). The nucleotide sequences and the amino acid sequences of α-amylases from *Aspergillus* sp. have been reported (Tada *et al.*, 1989; Wirsel *et al.*, 1989; Korman *et al.*, 1990), however almost all of these α-amylases cannot hydrolyze raw starch. The acid stable α-amylase from *A. kawachii* is the sole raw-starch-digesting α-amylase so far reported from *Aspergillus* sp. (Kaneko *et al.*, 1996). As for industrial application, raw-starch-degrading amylases are commercially important enzymes in the beverage, food, and textile industries. Raw-starch-digesting α-amylase from *Aspergillus* sp. is hardly used for industrial application until now, but is considered to become an effective tool.

We previously reported that *Aspergillus awamori* KT-11, a black mold which was isolated from Indonesia air, produced three kinds of amylolytic enzymes (Anindyawati *et al.*, 1998b). They were identified as α-amylases (named Amyl I, Amyl II and Amyl III), glucoamylase (GA I) and α-glucosidases (Anindyawati *et al.*, 1998a). Among these isolated enzymes, Amyl III had a remarkable starch digesting activity. Interestingly, this enzyme was found to have a molecular weight much bigger than other α-amylases form *Aspergillus* sp. Thus we tried to perform the cloning of the different encoding genes of the previously reported amylolytic

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enzymes, Amyl I, Amyl III, and GA I from *A. awamori* KT-11 to know the relationship between their structures and functions.

We purified Amyl I, Amyl III, and GA I from koji culture, and determined their N-terminal and internal amino acid sequences. We also succeeded the cloning of the cDNA of these different amylolytic enzymes from *A. awamori* KT-11.

This paper describes the nucleotide sequences encoding Amyl I, Amyl III, and GA I and their amino acid sequences. We reported also a comparison between the primary structures of these enzymes and we focus on the ability of Amyl III to hydrolyze raw starch based on its primary structure.

Materials and Methods

Chemicals Tryptone and yeast extract were purchased from Difco Co. Ltd. (Sparks, USA), Arginylendopeptidase, restriction endonucleases were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). pT7Blue Perfectly Blunt Cloning Kit from Novagen (Madison, USA), Quick Prep Micro mRNA Purification Kit were purchased from Amersham Pharmacia Biotech (Piscataway, USA). SMART RACE cDNA Amplification Kit was purchased from Clontech Co. Ltd. (Palo Alto, USA) Lysylendopeptidase and other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Strains and plasmid *A. awamori* KT-11 was used as a DNA source in this study. *Escherichia coli* TunerTM (DE3) pLacI and plasmid pT7Blue were used for general DNA manipulations and for DNA sequencing.

Culture media Wheat bran medium was used for the cultivation of *A. awamori* KT-11. It was consisted of 500 g of wheat bran (Miyake Flour Milling Co., Ltd., Osaka, Japan) and 500 ml of tap water in a pan (32 × 21.5 × 8.5 cm in size, 3 cm in thickness). LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) was used for *E. coli* cultivation.

Measurement of enzyme activities and protein concentration Soluble-starch-hydrolyzing activity (for α-amylase or glucoamylase) was carried out by incubating the mixture of enzyme (0.5 ml) and 0.25% of soluble starch (0.5 ml) in 50 mM acetate buffer (pH 4.8) at 37°C for 10 min. The amount of reducing sugar formed was determined by the Somogyi-Nelson method (Somogyi, 1951). One unit of the activity was defined as the amount of enzyme which released 1 μmol reducing sugar as equivalent to glucose per minute under these conditions. α-glucosidase activity was carried out using 0.25% of maltotoriitol as substrate under the same conditions as the soluble-starch-hydrolyzing activity. Raw-starch-hydrolyzing activity was assayed using 1.25% of raw corn starch as a substrate under the same conditions as for the soluble-starch-hydrolyzing activity. The protein concentration was determined by measuring the absorbance at 280 nm on a spectrophotometer U-1100 (Hitachi, Tokyo, Japan) by assuming the absorbance of 1% enzyme solution was 10.0.

Purification of Amyl I, Amyl III, and GA I *A. awamori* KT-11 was cultured in wheat bran medium for 5 d at 27°C. Amyl I and Amyl III were purified from the culture filtrate according to the procedures described by Anindyawati *et al.* (1998a) with a slight modification. On the other hand, GA I was purified as follow. The extract of fungus was further precipitated with 0.9 saturation of ammonium sulfate. The resulting precipitate was dissolved in deionized water and concentrated on hollow fibers. The solution was purified by successive chromatographies on Sephadex G-100 in 50 mM acetate buffer, pH 4.5, Hydroxyapatite in 5 mM potassium phosphate buffer, pH 7.1, DEAE Toyopearl 650 M, Toyopearl HW-55F, Butyl Toyopearl, and then Bio-Gel A0.5 in 50 mM acetate buffer, pH 4.5. All purified enzymes showed homogeneity in chromatography.

Analytical methods of SDS-PAGE Sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) was performed according to the method of Laemmli (1970). The molecular weight markers were purchased from Amarsham Pharmacia Biotech. Proteins were stained with Coomassie Brilliant Blue R-250.

N-terminal and partial amino acid sequences Proteins separated on SDS-PAGE gel were transferred onto PVDF membrane by electroblotting. The protein bands were cut out and were sequenced on an ABI 476A protein sequencer (Applied Biosystems Co. Ltd., Foster city, USA). Proteins were digested with arginylendopeptidase and lysylendopeptidase and the resulting peptides were fractionated on SDS-PAGE similarly.

Preparation of mRNA *A. awamori* KT-11 was cultured in wheat bran extract medium (pH 6.0) for 48 h at 27°C with shaking. Mycelia harvested on the filter membrane by filtration were immediately frozen in liquid nitrogen and pulverized. The cell powder was suspended in 4 M guanidine isothiocyanate. The mRNA was isolated from the solution with Quick Prep Micro mRNA Purification Kit.

Cloning of cDNA First strand cDNA fragment was synthesized from mRNA with SUPERSCRIPT II RT (Sigma, St. Louis, USA) using oligo $(dT)_{20}$ as the primer and cDNA fragment. For 5'- and 3'-Rapid amplification of cDNA ends PCR (RACE) was synthesized with SMART RACE cDNA Amplification Kit according to the manufacture's protocol. Oligonucleotide primers for PCR were synthesized on the basis of the N-terminal and partial amino acid sequences. Reverse transcription PCR (RT-PCR) products were subcloned into plasmid pT7Blue. Nucleotide sequences of PCR fragments were analyzed by a DNA sequencer (ABI PRISM 310).

Computer analysis The analysis, and the translation of the nucleotide sequences were performed with the GENETYX Mac (Software Development). A homology search and alignment of amino acid sequences were done with the FASTA and BLAST programs.

Nucleotide sequence accession number The DNA sequences of Amyl III, Amyl I, and GA I have been deposited in DDBJ under the accession no. AB083159, AB083160, and AB083161, respectively.

Analytical methods of sugar composition and PAS staining The analysis of glycomoiety of glycoprotein was performed as follows. The sugar composition of Amyl III was determined using the method described by Gerard (1990). Amyl III was electrophoresed on a 10% acrylamide gels and stained for glycoproteins with the Periodic Acid-Schiff (PAS) stain. Analysis of carbohydrate composition was performed as follows. The carbohydrate composition of Amyl III was determined using HPLC

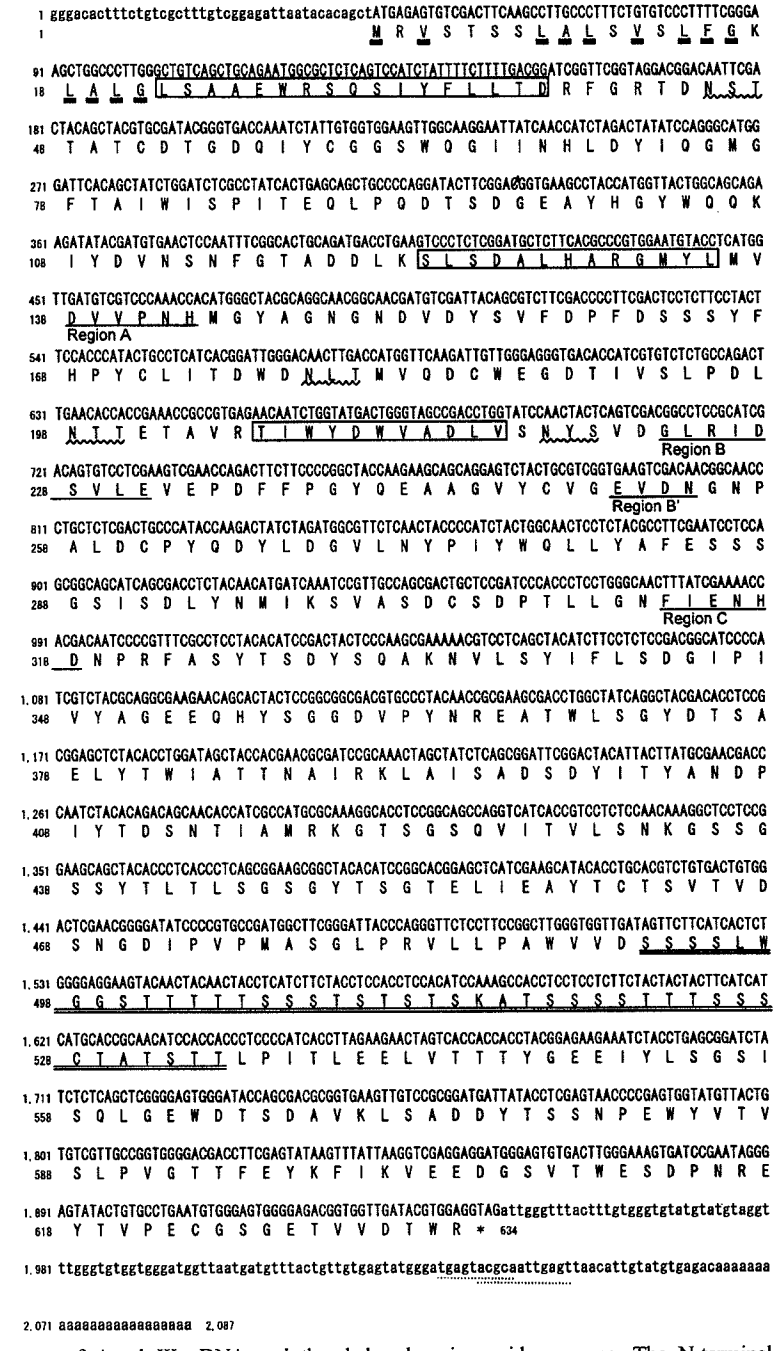

Fig. 1. Nucleotide sequence of Amyl III cDNA and the deduced amino acid sequence. The N-terminal and internal amino acid sequences of Amyl III are boxed. Bold lines indicate hydrophobic amino acid residues in the signal peptide. Region A, B, B' and C indicate conserved regions of α-amylase. Wave lines indicate the sites possible to combine with sugar chain. Double line indicates TS region. Broken lines indicate palindrome structure.

according to the method of ABME. Amyl III was hydrolyzed with 4 M hydrochloric acid at 100°C, and the hydrochloric acid was removed under reduced pressure. Thereafter acetic anhydride and sodium bicarbonate was added and incubated at room temperature for 10 min, and this sample was concentrated after desalting by passing through a column of Dewex 50W-X8(H+) (BioRad, Hercules, USA). This sample was dissolved in water, and applied to column SepPak (Waters), and eluted with water. The elution was dried up under airflow of nitrogen, and methylated with the reagent containing p-aminobenzoic acid. After drying, the sample was dissolved in water, and the sugar composition analysis was performed using the column TSK-GEL ODS-120T (Tosoh Co., Ltd., Tokyo, Japan) with the authentic markers (galactose, mannose, fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine).

Result

Amino acid sequence analysis of peptides from Amyl III, Amyl I, and GA I N-terminal amino acid sequence of Amyl III was determined chemically and was found to be LSAAEWRSQ. Since the N-terminal amino acid was not methionine but leucine, it turns out that N-terminal of Amyl III was processed. When the identified N-terminal amino acid sequence was compared to the existing sequences in the database, acid α-amylase of *A. niger* (Korman *et al.*, 1990) showed 7 identical residues. Internal amino acid sequences of the fragments produced by peptidase digestion of Amyl III were TITYDWDADLV and SLSDALHRGMWL. These

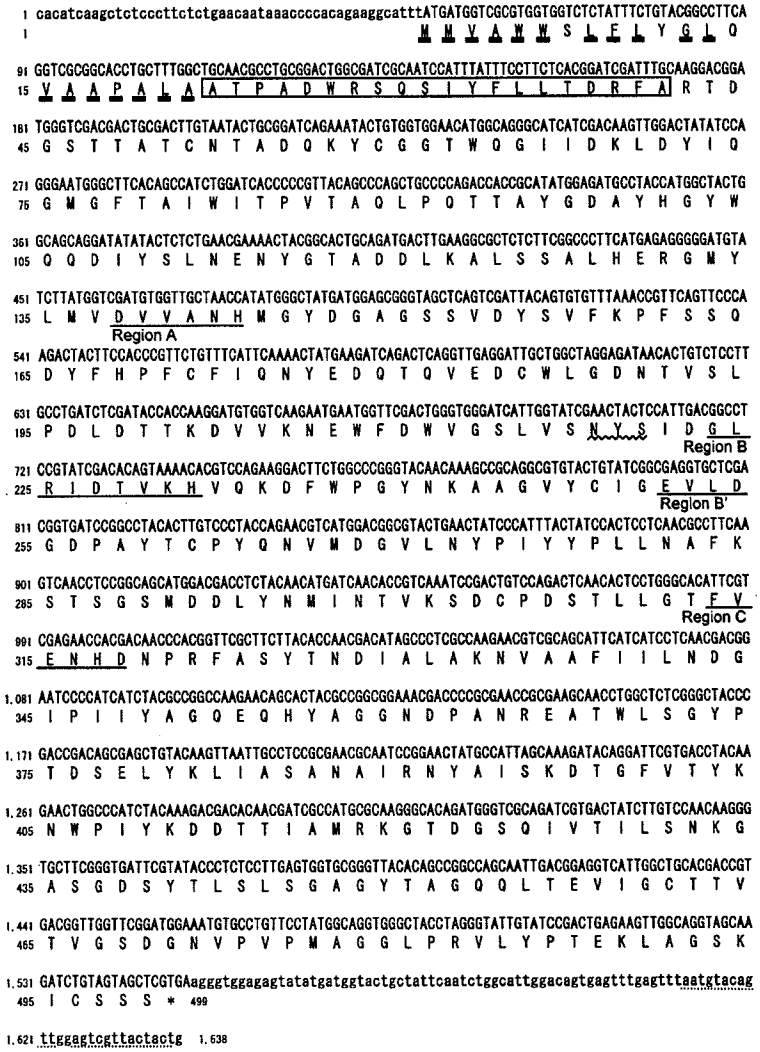

Fig. 2. Nucleotide sequence of Amyl I cDNA and the deduced amino acid sequence. The N-terminal amino acid sequence of Amyl I are boxed. Bold lines indicate hydrophobic amino acid residues in a signal peptide. Region A, B, B' and C indicate conserved regions of α-amylase. Wave line indicates the sites possible to combine with sugar chain. Broken lines indicate palindrome structure.

sequences had a significant degree of homology with the acid α-amylase from *A. niger* and the Taka-amylase A from *A. oryzae* (Tada *et al.*, 1989). N-terminal amino acid sequence of Amyl I was ATPADWRSQSIYFLLTDKFA. This sequence has 19 residues homologous with that of the Taka-amylase from *A. oryzae*. However, only 13 residues were homologous with that of the acid α-amylase from *A. niger*. N-terminal amino acid sequence of GA I was ATLDSWLSNEATVARTA.

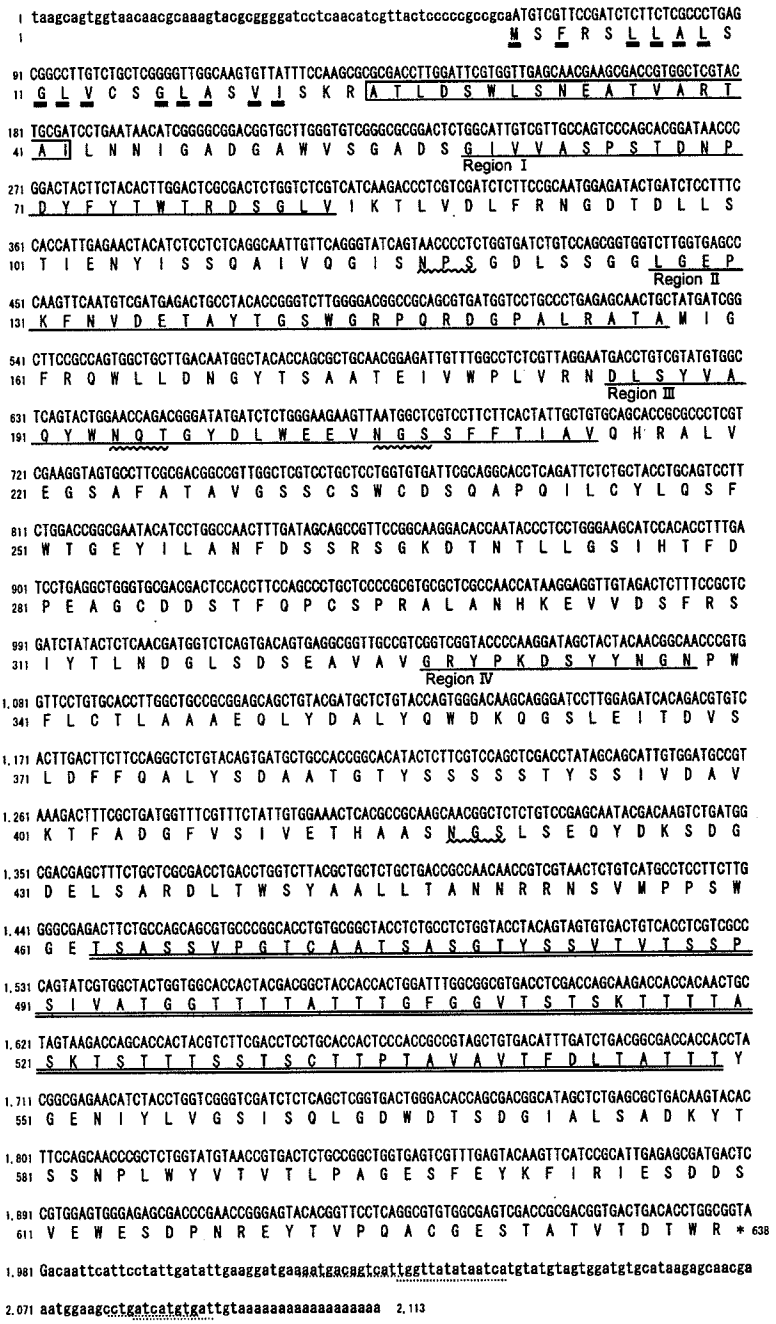

Fig. 3. Nucleotide sequence and the deduced amino acid sequence of GA I cDNA. The N-terminal amino acid sequence of GA I are boxed. Bold lines indicate hydrophobic amino acid residues in a signal peptide. Region I, II, III and IV indicate conserved regions of glucoamylase. Wave lines indicate the sites possible to combine with sugar chain. Double line indicates TS region. Broken lines indicate palindrome structure.

This sequence showed complete homology with glucoamylase from *A. awamori* (Nunberg *et al.*, 1984), *A. niger* (Boel *et al.*, 1984) and *A. shirousamii* (Shibuya *et al.*, 1990). However, only 11 residues were homologous with that of glucoamylase from *A. oryzae* (Hata *et al.*, 1991).

Nucleotide sequence of Amyl III cDNA Based on the N-terminal and internal amino acid sequences of Amyl III, oligonucleotide PCR primers were designed; Amyl III-4F primer (5'-CTGTCAGCTGCAGAATGGCG-3') and Amyl III-2R primer (5'-TCCCAAGTCACACTTCCACC-3'). Using mRNA as a template, 3'-RACE cDNA fragment was synthesized using Amyl III-4F as a sense primer. 5'-RACE cDNA fragment was synthesized using Amyl III-2R as an anti sense primer. The full length cDNA of Amyl III was achieved by primer walking method. The Amyl III nucleotide sequence, except for poly (A) sequence, and the deduced amino acid sequence from the full length cDNA are shown in Fig. 1. The sequence of 2,087 nucleotides, showed an open reading frame from a start codon (ATG), at position 42 bp to a stop codon (TAG) at position 1,943 bp. This ORF encodes a polypeptide of 633 amino acid residues. A putative polyadenylation signal, AATAAA, was not found. Moreover, two palindrome structures existed in the trailer sequence. The N-terminal residue of the purified enzyme was the amino acid number 22 (leucine) from the N-terminal of the primary structure presumed from cDNA. The sequence from amino acid number 22 to 38 (17 residues) was completely in agreement with N-terminal amino acid sequence of the purified enzyme determined by the Edman method. Since the amino acid sequence of the protein was confirmed in cDNA, it was considered that it was the clone coding for Amyl III. As the starting amino acid in the purified enzyme corresponded to number 22 (leucine) in the deduced amino acid sequence, it was thought that the sequence from amino acid number 1 to 21 was signal peptide. Among the 615 amino acids consisting the mature protein, hydrophobic amino acids represent 13 residues within the 21 residues of the signal peptide. Moreover, it was found that among the 43 residues of TS domain, referred to the domain from amino acid number 492 to 534, 35 residues were serine or threonine.

Nucleotide sequence of Amyl I cDNA Taking advantage the high homology between the N-terminal amino acid residues of Amyl I and that of α-amylase from *A. awamori*, the oligonucleotide primers used for the cloning of Amyl I encoding cDNA were synthesized. The cloning of Amyl I was performed as same as the case of that of Amyl III. The Amyl I nucleotide sequence and the deduced amino acid sequence of full length cDNA are shown in Fig. 2. The sequence of 1,638 nucleotides showed the ORF from a start codon (ATG) at position 50 bp to a stop codon (TAG) at position 1,546 bp. This ORF encodes a polypeptide of 499 amino acid residues. A putative polyadenylation signal, AATAAA, was not found. Moreover, two palindrome structures existed in the trailer sequence. The amino acid sequence from amino acid number 22 to 41 corresponds to that of the N-terminal region of the peptide derived by endopeptidase. Therefore, the sequence from amino acid number 1 (methionine) to 21 (alanine) was considered to be the signal peptide and the mature protein consists of 478 amino acids.

Nucleotide sequence of GA I cDNA Oligonucleotide primers for GA I cDNA cloning were designed as refer to glucoamylase from A. awamori, because the N-terminal amino acid residues of GA I agreed with glucoamylase from *A. awamori*. The GA I nucleotide sequence and the deduced amino acid sequence of full length cDNA are shown in Fig. 3. The sequence of 2,113 nucleotides showed the ORF from a start codon (ATG) at position 62 bp to a stop codon (TAG) at position 1,978 bp. This ORF encodes a polypeptide of 639 amino acid residues. A putative polyadenylation signal, AATAAA, was not found. Moreover, two palindrome structures existed in the trailer sequence. The amino acid sequence from amino acid number 25 to 42 corresponds to that of the N-terminal region. Therefore, the sequence from amino acid number 1 (methionine) to 24 (arginine) was considered to be the signal peptide and the mature protein consists of 615 amino acids. The amino acid sequence from amino acid number 463 (serine) to 549 (threonine) was identified as TS region that was rich on Threonine and Serine.

Amino acid sequence similarity The amino acid sequence of Amyl III has 93% homology with the acid α-amylase from *A. niger* (Korman *et al.*, 1990). On the other hand, the homology with α-amylase from *Aspergillus* sp. was 66% (Tada *et al.*, 1989). The molecular weight of Amyl III was

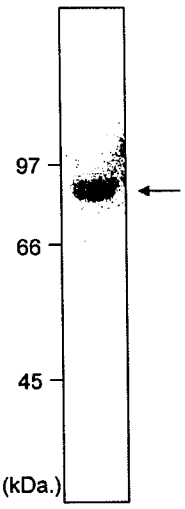

Fig. 4. PAS stain of Amyl III. Ten μg of Amyl III purified from *A. awamori* KT-11 was loaded on 10% polyacrylamide gel. After electrophoresis, carbohydrate on the gel was stained by the methods described in the text.

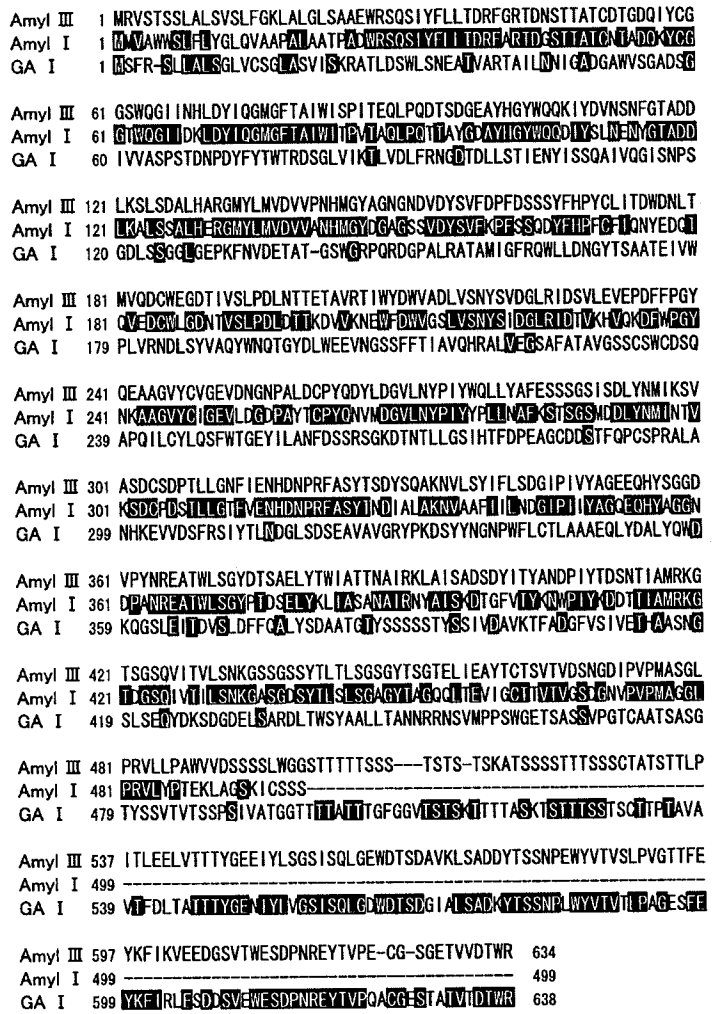

Fig. 5. Alignment of amino acid sequences of Amyl I and GA I with Amyl III. The putative amino acid sequence of Amyl III was compared with that of Amyl I and GA I. Amino Acid residues of Amyl III identical to that of Amyl I or GA I are boxed.

about 70 kDa. However, the molecular weight of the α-amylases from *Aspergillus* sp. is about 50 kDa. The place which performed alignment of the amino acid sequence of Amyl III and acid α-amylase of *A. niger* which has the highest similarity, among the 484 residues of acid α-amylase of *A. niger*. Although the 444 residues was mostly in agreement with the domain from N-terminal which received processing of Amyl III to 505, the domain which is from the amino acid of a 129 residues on C-terminal side further existed in Amyl III. The amino acid number 229 in the B-Region (consensus sequence of the α-amylase family) was glutamic acid in Amyl III, however it is histidine in almost all the other α-amylases. Moreover, in the B'-region of Amyl III, the amino acid number 253 was found to be a hydrophobic amino acid (aspartic acid), however it was hydrophilic amino acid (leucine, tryptophan, and isoleucine) in all the other α-amylases. Furthermore, the amino acid number 254, which was aspartic acid, glutamine, and serine in most other α-amylases, was replaced by asparagine in Amyl III.

The amino acid sequence of Amyl I has 99% homology with α-amylase from *A. oryzae* (Tada *et al.*, 1989). It is known that the amino acid sequences of the four domains responsible for an active center of α-amylase family is highly (preserved). When compared with the amino acid sequence of these domains, the sequences of A-region, B-region, B'-region, and C-region of Amyl I were mostly in agreement with consensus sequences.

The amino acid sequence of GA I was similar to glucoamylase from *A. shirousamii* (Shibuya *et al.*, 1990) and *A. niger* (Boel *et al.*, 1984) with arrange of homology from 95 to 97%. It is also known that the amino acid sequences of the four domains that form the active center is highly conserved at glucoamylases. All the sequences of region I to IV of other glucoamylases were completely in agreement with the

consensus sequences of GA I.

Analysis of glycomoiety of glycoprotein as Amyl III The PAS staining of Amyl III was shown in Fig. 4. The clear band was found in the same position as the one obtained by CBB staining and the molecular weight was 90 kDa. This resulted to the conclusion that Amyl III is a glycoprotein enzyme. In carbohydrate composition analysis, Amyl III contains mannose and *N*-acetylglucosamine at 462.86 and 72.86 μmol per one mol of protein, respectively. However, fucose and *N*-acetylgalactosamine were not detected. Consequently, it was presumed that *N*-glycosidic linkage exists in the sugar chain of Amyl III, and *O*-glycosidic linkage could not be detected. On the other hand, it was presumed that this sugar chain was high mannose type, because the ratio of Man/GlcNAc was 6.3, which is considered as higher value.

Discussion

Amyl III and Amyl I had a clear difference in their primary structures. Amyl III was 135 residues longer than Amyl I. Using Harrplot analysis to compare the amino acid sequences of Amyl III and Amyl I and GA I, it was found that the amino acid sequences of Amyl III and Amyl I showed a good correlation of 63.3% of identity along the region from the N-terminal to the amino acid number 420 (Fig. 5). However, Amyl III and GA I showed a high correlation 66.9% of identity only along the region from the amino acid number 450 to C-terminal. No correlation was found between Amyl I and GA I.

From the above results we can conclude that Amyl III seems to be a hybrid of α-amylase and glucoamylase as illustrated in Fig. 6. It contains the N-terminal region of α-amylase family and C-terminal region of glucoamylase family.

Amyl III showed a domain rich in threonine and serine. This domain consisted of 43 residues and was located between the amino acid number 429 and 534. The latter region called TS domain was also found in GA I.

Nunberg reported that raw starch adsorption domain of glucoamylase is located near the C-terminal region, and that the TS domain serves as hinge portion between the N-terminal and the C-terminal regions (Nunberg *et al.*, 1984). α-amylases of *Aspergillus* sp., including Amyl I, do not have TS domain and they are unable to digest raw starch. The presence of a C-terminal region in Amyl III similar to that of GA I, which is able to digest raw starch, can explain the capability of Amyl III to adsorb into raw starch.

Although the TS domain of Amyl III was shorter than that of GA I, the similarity between the two domains was very high. It was reported that the length of TS domain of glucoamylase influence the raw starch digesting activity (Semimaru *et al.*, 1995). Raw starch digesting activity of Amyl III may rise by adjusting the distance between the two

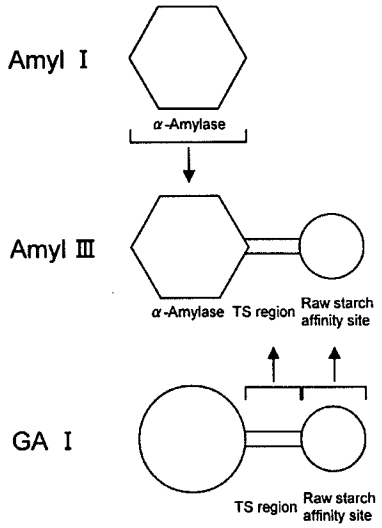

Fig. 6. Proposal of structure and function of Amyl III.

domains.

The molecular weight of Amyl III was 90 kDa. as determined by SDS-PAGE. However, the molecular weight deduced from the amino acid sequence (without signal peptide) was 67,081 Da. The difference between the two molecular weights can be explained by the existence of carbohydrate in Amyl III. The amount of carbohydrate as measured by Phenol sulfuric acid method was 11.3%. Since Amyl III is modified by oligosaccharides linked by *N*-glycosidic linkage, and fucose was not detected when sugar composition analysis was performed, it became clear that Amyl III has oligosaccharides linked with *N*-glycosidic and not with *O*-glycosidic linkages. It was reported that glucoamylase from *Aspergillus* sp. had *O*-glycosidic linked oligosaccharides attached to the TS domain (Gunnarsson *et al.*, 1984; Hayashida *et al.*, 1989; Williamson *et al.*, 1992). It is known that oligosaccharides in glycoproteins are involves in the maintenance of the protein structure against stress caused by heat, pH, and pressure and also involved in secretion of protein out of cells (Dube *et al.*, 1988; Chen *et al.*, 1994; De Cordt *et al.*, 1994). However, Amyl III did not have these *O*-glycosidic linked oligosaccharides. Physical property of Amyl III, such as the pH stability and heat stability, is almost the same as Amyl I. However, raw starch digesting activity is low as compared with GA I. The absence *O*-glycosidic linkage in Amyl III may have caused the low activity of few enzymes on raw starch. Otherwise, the tertiary structure of Amyl III might be different from that of GA I.

In conclusion, cDNA fragment that encodes an α-amylase (Amyl III) with raw starch digesting activity was cloned from *Aspergillus awamori* KT-11. In addition, the cDNA fragments encoding for typical α-amylase (Amyl I) and glucoamylase (GA I) were also cloned from the same strain. The primary structures of the enzymes were compared. Amyl III was a

hybrid type α-amylase, which has a catalytic domain similar to that of α-amylase family located in the N-terminal region, and a binding domain in the C-terminal region similar to that of glucoamylase having a raw starch affinity. The two domains are linked by a TS domain rich in Threonine and Serine.

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